

Experimental Group B Streptococcal Infections in Mice: Hematogenous Virulence and Mucosal Colonization

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A group B streptococcus recovered from a blood specimen from a neonate with sepsis was used to evaluate the use of mice for studies characterizing the hematogenous virulence and the asymptomatic mucosal colonization of the vagina or of the respiratory tract by these bacteria. When injected intravenously, the 50% lethal dose for mice was 10^6 ; however, as few as 10^2 organisms produced septic deaths. In mice undergoing water diuresis, bacteriuria and pyelonephritis were not produced after direct bladder inoculation of the streptococci. Asymptomatic vaginal colonizations that persisted for 12 days were produced in both pregnant and virgin mice. Vaginal colonization before delivery did not result in transmission of infection to litters or in protection against subsequent oropharyngeal colonization in the suckling mice. In mice born of nonexposed mothers, oropharyngeal colonization was produced in both suckling and 3-week-old weaned mice. Whereas infection persisted for 14 days in all suckling mice, clearance occurred in over 50% of the weaned mice by day 14. The use of mice for studies on the virulence of the group B streptococci as well as for studies on the pathogenesis of disease by virulent strains is discussed.

The group B beta-hemolytic streptococci have recently emerged as major etiological agents of neonatal sepsis and meningitis. Recent studies have documented the incidence of asymptomatic vaginal infections among pregnant women as ranging from 4.6 to 25.4% (1, 4, 7, 14, 15). The rate of maternal transmission of organisms to the neonate has been reported to be 1.2 to 26.2% (1, 7, 15). Because organisms have been recovered from the umbilicus, ear, and nasopharynx, the route by which the organisms gain access to these sites has been postulated to be either by ascending intrauterine infection or by exposure in passage through the birth canal (3, 4, 17). In all of these studies the incidence of neonatal disease, acute onset sepsis or delayed onset meningitis, consistently has been reported to be 0.2 to 0.3% (1, 23). Despite vigorous treatment with the appropriate antibiotics, the mortality rates associated with disease remain high, that is, 40 to 80% (16, 18, 19). The pathogenesis of neonatal invasive disease and the basis for these treatment failures are not understood.

These clinical observations, high colonization rates with low disease production, have resulted in the discussion of prophylactic antibiotic treatment as a preventive measure (5, 8, 20). However, before routine prophylactic treatment is recommended, documentation of the

effectiveness of treatment in eradicating maternal vaginal infections, in preventing transmission and colonization of the neonate, or in preventing neonatal disease is needed.

Studies were undertaken in our laboratory to evaluate the use of mice for examining the virulence of a group B streptococcus recovered from the blood of a neonate with sepsis. Because this strain produced a fulminating sepsis after intravenous injection, experiments were carried out to evaluate the efficacy of establishing vaginal and oral mucosal colonizations in mice without production of fulminating invasive disease. The use of this experimental model for studies on the pathogenesis of invasive disease as well as the effectiveness of antibiotic treatment for eradicating mucosal colonization and/or preventing sepsis are discussed.

MATERIALS AND METHODS

Bacteria. The group B streptococcus isolate was recovered from a blood specimen of a septic neonate. The organism was beta-hemolytic, did not grow on bile esculin, hydrolyzed sodium hippurate, was resistant to bacitracin, and was sensitive to penicillin, ampicillin, and cephalosporin. Using specific antisera and the standard capillary precipitin reaction, the serotype of this isolate was B₁₁. Stock cultures were maintained on blood agar plates and stored at 4 C. A 3-h log phase culture grown in Todd-Hewitt broth containing 5% sheep blood was diluted in

physiological saline for inoculation. Bacterial counts were made using the standard surface plating technique.

Mice. Cornett strain mice, weighing 20 to 25 g, were used for the hematogenous, urinary tract, and vaginal routes of infection. For studies on the colonization of the oral mucosa, 4- to 7-day-old suckling and 3-week-old weaned mice were used.

Hematogenous infections. A 0.5-ml volume of serially diluted log-phase cultures was inoculated into a lateral tail vein. Deaths were recorded as cumulative through day 5 or 7. Surviving mice were sacrificed, and the kidneys and spleen were removed, homogenized, and quantitatively analyzed for streptococci by direct surface plating on blood agar plates. Short-term *in vivo* growth studies were carried out as previously described (9), with blood, spleen, and kidney tissues analyzed for bacteria at the indicated times. The blood specimens were obtained by orbital sinus bleedings before sacrifice.

Ascending urinary tract infections. To measure the ability of this isolate to produce experimental bacteriuria, the water diuresis model characterized by our laboratory in previous studies for *Escherichia coli* and *Staphylococcus aureus* was used (10). The 10^8 streptococcal inoculum was introduced directly into the bladder lumen. Mice were observed for 7 days, after which the animals were sacrificed and the urine, kidneys, and spleen were analyzed for the recovery of infecting organisms.

Vaginal infections. Groups of female mice, virgin or pregnant, were inoculated in the vaginal orifice by rotating a small cotton swab dipped in a broth culture containing 10^{10} group B streptococci per ml. The inoculum contained 10^6 organisms. This inoculation procedure was repeated on 3 consecutive days. Mice were observed for 7 days, and vaginal specimens were taken on days 4 and 7 after the last inoculation. The swabs were incubated overnight at 37 C in 1.0 ml of Todd-Hewitt broth containing 5% sheep blood and then subcultured on blood agar plates for identification. The results were interpreted as positive or negative without quantitative evaluation.

Throat infections. Groups of suckling or weaned mice received a 0.02-ml inoculum of group B streptococci (10^8 bacteria) delivered by micropipette directly into the mouth. This inoculation procedure was repeated on 3 consecutive days. Using saline-moistened swabs, throat specimens were taken on days 7 and 14 after the last inoculation, and the specimens were processed as described for vaginal specimens.

RESULTS

Hematogenous infections. Groups of 10 to 24 mice were intravenously inoculated with 0.5 ml of 10-fold serial dilutions containing 10^7 to 10^2 organisms. The mortality rate was a dose response (Table 1), being highest (75%) for the highest dose (3.2×10^7 streptococci). The sepsis was fulminating, with deaths occurring between 2 and 5 days. Of the 6 mice sacrificed on

day 7, bacteriological analysis of the kidneys and urine showed that four animals had infections. The recovery of organisms from the urine indicated that renal abscesses had been produced. With reduction of the inocula to 1.5×10^6 , the mortality rate declined to 50%, as did the infection rate with streptococci recovered from just one of the six urine specimens and from two of the six pairs of kidneys. Decreasing the dosage to 10^4 or 10^3 organisms further reduced both the mortality and infection rates. The production of one or two septic deaths after inoculation of 10^2 or 10^3 organisms placed the virulence of the streptococci for mice as greater than what we have observed after intravenous inoculation of *Pseudomonas aeruginosa*, *E. coli*, or *S. aureus* (9, 12; D. Furtado, submitted for publication). In addition, organisms were recovered from the spleen of some of the surviving mice, indicating a persistent hematological infection through day 7. From these data the 50% lethal dose for this strain of group B streptococci was calculated to be a $10^{-2.4}$ dilution of a log-phase culture or 1.2×10^6 organisms.

The incidence of streptococcal renal infection among surviving mice was considerably lower than that reported after intravenous inoculation of *Staphylococcus aureus* or *Streptococcus faecalis* (13), but was more similar to the incidence seen after inoculation of *E. coli* or *P. aeruginosa* (12). Because Gorrill et al. (13) concluded that the renal disease-producing capability of bacteria after intravenous inoculation could be accurately predicted in mice through the pattern of early *in vivo* growth in the kidneys, experiments were undertaken to characterize the pattern of *in vivo* growth of group B streptococci in the kidneys. Quantitative monitoring of the bacteremia and spleen was also included.

TABLE 1. Dose response to intravenous injection of Group B streptococci^a: mortality and morbidity in mice

Dose	Deaths ^b	Recovery of organisms from tissues of survivors at sacrifice ^c		
		Spleen	Kidneys	Urine
3.2×10^7	18/24	ND ^d	4/6	4/6
1.5×10^6	4/10	1/6	2/6	1/6
7.5×10^5	4/11	2/7	2/7	1/7
7.5×10^4	5/12	1/7	1/7	0/7
1.3×10^3	2/12	2/10	2/10	2/10
1.3×10^2	1/12	0/11	0/11	0/11

^a 0.5 ml injected into lateral tail vein.

^b Cumulative deaths through day 7.

^c Tissue homogenate or urine specimen positive for group B streptococci on day 7.

^d ND, Not done.

Eight mice were sacrificed at each of the indicated times, immediately after inoculation and at hourly intervals up to 8 h after inoculation. The mean number of organisms deposited in the kidneys after intravenous inoculation of 1.0×10^7 streptococci was 3.5×10^5 (Table 2). This number was similar to the numbers of bacteria, gram positive and gram negative, that are consistently deposited in the kidneys after intravenous inoculation of 10^7 organisms (9, 11; Furtado, submitted for publication). Between 1 and 4 h after inoculation, there was a continuous decline and maintenance of 10^3 organisms, which was followed by a slight increase at 6 and 8 h. In the blood, 5.7×10^6 streptococci per ml were recovered immediately after inoculation, followed by rapid clearance by 2 h to 10^3 organisms per ml of blood. Once again, the numbers of bacteria persisting in the blood at 3, 4, 6, and 8 h remained at a 10^3 /ml level, indicating that the host was not able to clear the blood stream and that proliferation kept up with any host defenses.

In a recent study in our laboratory, the level of bacteremia in dogs continuously infused for 2 h with 10^6 *P. aeruginosa*/ml per min actually declined during infusion and total clearance occurred within 2 h after termination of the infusion (22). Fisher et al. (6) reported that in rabbits, blood clearance after a bolus injection of 10^8 *E. coli* occurred in 30 min. Therefore, it appears that mice are unable to clear the bloodstream of the streptococci, which may reflect the hematogenous virulence of this particular strain.

The numbers of organism recovered from the spleen showed that at 1 and 2 h after inoculation there were 10^6 and 10^5 streptococci in the spleen. Throughout the remainder of the observation times the number remained at 10^4 , which was a log higher than the numbers of

organisms recovered per pair of kidneys or per milliliter of blood.

In Fig. 1 the pattern of in vivo growth of the group B streptococci in the kidneys is compared with our previously reported results using *S. aureus* and *E. coli* (9; Furtado, submitted for publication). It can be seen that the continuous decline in the kidneys over 4 h more closely resembled the pattern seen using *E. coli*. After the intravenous inoculation of *S. aureus*, an organism that caused hematogenous pyelonephritis in virtually 100% of the mice, the numbers of organisms in the kidneys increased after 2 h.

Bacteriuria and ascending urinary tract infections. Because it has been suggested that the group B streptococci should be considered as potential urinary tract pathogens for man (7, 22), experiments were undertaken to evaluate the pathogenicity of these bacteria for mice undergoing water diuresis.

Using the direct bladder route of inoculation, 13 mice received 10^3 streptococci. At sacrifice on day 7, none of the mice had developed a $>10^2$ /ml

TABLE 2. In vivo proliferation of group B streptococci^a in the blood, spleen, and kidneys of mice^b

Time (h)	Number of bacterial recovered ^c per		
	Blood (ml)	Spleen (ml)	Kidneys (ml)
0	$5.7 \times 10^6 \pm 0.3$	$1.2 \times 10^5 \pm 0.3$	$3.5 \times 10^5 \pm 0.3$
1	$1.6 \times 10^4 \pm 0.6$	$2.4 \times 10^6 \pm 0.4$	$1.0 \times 10^4 \pm 0.3$
2	$1.5 \times 10^3 \pm 0.4$	$4.1 \times 10^5 \pm 0.5$	$7.8 \times 10^3 \pm 1.0$
3	$2.1 \times 10^3 \pm 0.9$	$7.6 \times 10^4 \pm 1.1$	$6.1 \times 10^3 \pm 1.2$
4	$1.9 \times 10^4 \pm 0.5$	$6.1 \times 10^4 \pm 0.4$	$5.3 \times 10^3 \pm 0.7$
6	$6.8 \times 10^3 \pm 1.4$	$1.5 \times 10^4 \pm 0.4$	$9.3 \times 10^3 \pm 1.9$
8	$2.0 \times 10^3 \pm 0.7$	$1.8 \times 10^4 \pm 0.4$	$1.4 \times 10^4 \pm 0.3$

^a A 0.5-ml amount containing 1.0×10^7 organisms injected into lateral tail vein.

^b Eight mice sacrificed at each time interval.

^c For each interval, mean number \pm standard error of the mean.

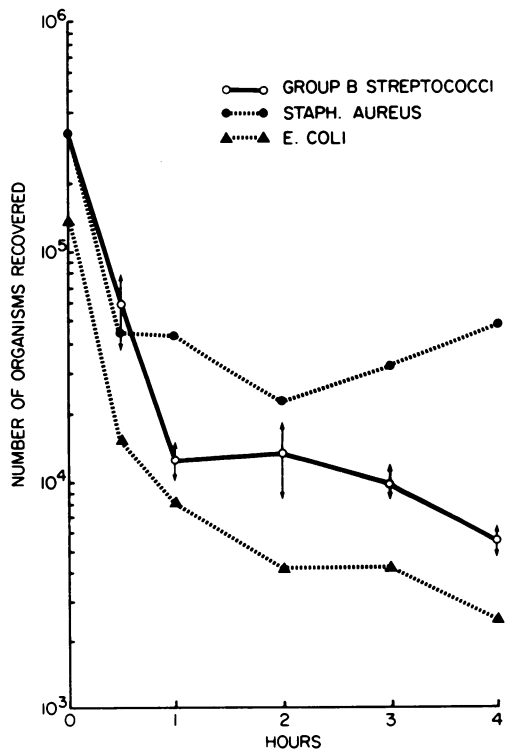


FIG. 1. In vivo growth of hematogenously delivered group B streptococci, *Staphylococcus aureus*, and *E. coli* in the kidneys of mice. Points are the mean number of bacteria recovered from the kidneys of 12 mice; bars are standard error of mean.

bacteriuria or retrograde infection of the kidneys. In comparable experiments using 10^3 *E. coli*, 50 to 80% of the mice developed 10^6 bacteriuria and retrograde pyelonephritis by day 7 (10). Although 10^2 to 10^3 streptococci persisted in the urine, kidneys, and spleens of four animals, six mice had completely cleared the inoculum. In no instance were organisms recovered from the urine alone. Fatal bacteremia associated with the surgical procedure occurred within 2 days in three mice. We have not observed such a complication after intrabladder inoculation of *E. coli*, *P. aeruginosa*, or *S. aureus*. This suggests that the unavoidable introduction of even a few organisms into the peritoneal cavity and/or bloodstream resulted in sepsis in the surgically manipulated host.

The results of this experiment further support the thesis that these organisms are highly virulent by the hematogenous route and that this is not by retrograde invasion or infection of the urinary tract.

Vaginal infections. Vaginal infections in women appear to be noninvasive asymptomatic infections. Therefore, to establish colonization of the vaginal mucosa of mice, 10^8 streptococci were inoculated into the vaginal orifice of 24 pregnant mice on 3 consecutive days. There were no deaths recorded after inoculation or during the course of the observation period. As shown in Table 3, 17 of the 24 vaginal specimens were positive 4 days after the last inoculation. On day 7, 15 of the 24 were positive; however, superinfections with *Pseudomonas aeruginosa* and *Proteus* sp. were frequent. There were no maternal deaths associated with delivery of any litters. Newborn mice randomly selected for sacrifice did not have group B streptococci recovered from the lungs, spleen, or kidneys. One sick animal had a disseminated gram-negative sepsis.

Vaginal infections were similarly produced in virgin mice. Four days after the last inoculation, 18 of the 24 vaginal specimens were positive, which declined to 13 of the 24 by day 7 (Table 3). Superinfection with gram-negative bacilli was frequent on day 14. Overall there were no differences in the rate of vaginal colonization or in the persistence of infection between pregnant and virgin mice. Despite the use of repeated 10^8 inocula, bacteremia was not produced as a consequence of vaginal inoculation.

Throat infections. It has been suggested that one of the routes by which the nasopharynx of neonates is colonized is by aspiration of contaminated amniotic fluid (3, 4, 17). Attempts to experimentally produce oral mucosa colonization were made by using suckling mice born of mothers that had been vaginally ex-

posed to the group B streptococci and in suckling mice born of nonexposed mothers. A 10^8 inoculum was delivered by micropipette into the mouth on 3 consecutive days. On days 7 and 14, 18 of 18 or 100% of the throat cultures taken from suckling exposed mice were positive (Table 4). Positive specimens were also obtained from 51 of 52 suckling nonexposed mice on day 7 and from 43 of 44 animals on day 14. There was no difference in the rate of colonization or in the persistence of infection between these two groups.

When 3-week-old weaned nonexposed mice were similarly infected, 27 of 28 specimens were positive on day 7; on day 14, only 5 of 12 specimens were positive. Therefore, in older mice persistent infection occurred in 42% of the animals as compared with 98% of the suckling mice. There were no deaths due to bacteremia after oral inoculation. No group B streptococci were recovered from the lungs of randomly selected mice sacrificed on day 8 or 15.

DISCUSSION

Studies on the pathogenicity of a group B streptococcal isolate recovered from the blood of

TABLE 3. Group B streptococcal vaginal colonization^a in pregnant and virgin mice^b

Condition of mice	Recovery of group B streptococci in vaginal specimens ^c taken on:	
	Day 4	Day 7
Pregnant	17/24	15/24
Virgin	18/24	13/24

^a Inoculation of 10^8 organisms into the vaginal orifice by a rotating cotton swab dipped into broth culture.

^b Twenty-four animals in each group.

^c Swab specimen incubated overnight at 37 C in enrichment broth followed by subculture to blood agar plate.

TABLE 4. Group B streptococcal oral mucosa colonization^a in suckling and weaned mice

Age	Maternal ^b exposure	Recovery of streptococci in throat specimen ^c taken on day:	
		7	14
Suckling	+	18/18	8/8
Suckling	-	51/52	43/44
Weaned	-	27/28	5/12

^a Inoculation of 0.02 ml containing 10^8 organisms directly into mouth.

^b +, Litters from mice exposed to group B streptococcal vaginal inoculations during pregnancy; -, litter from nonexposed mice.

^c Swab specimen incubated overnight at 37 C in enrichment broth followed by subculture to blood agar plate.

a neonate with sepsis were undertaken in mice, an experimental animal that has been used in our laboratory for studies on the pathogenesis of bacterial infections of the kidneys and urinary tract.

Virulence in the bloodstream was supported (i) by the development of sepsis after intravenous inoculation of as few as 10^2 organisms, (ii) by the development of the bacteremia and death after intrabladder inoculation of 10^3 organisms, and (iii) by the failure of the mice to clear the bloodstream of inoculated organisms within 8 h. The *in vivo* pattern of growth in the kidneys suggests that the kidneys are not organs in which localized infection can be expected after an elevated bacteremia. Gorrill et al. (13) demonstrated that in mice the kidneys are the target organs for infection after intravenous injection of several different gram-positive and gram-negative genera of bacteria. Whereas high doses of gram-negative bacteria produced toxic-septic deaths within 24 h after inoculation (12), the pathogenesis of septic deaths after inoculation of this virulent strain of group B streptococci was slower, taking 2 to 5 days. Experimental fulminating bacteremia was produced by the higher doses; this may be compared to early onset disease in neonate sepsis, which also is rapidly fatal. Of course the clinical observations may not only be related to a specific subtype of streptococcus but may also be dose dependent, related to route of invasion, and involve consideration of the immune capabilities of the host. Klesius et al. (18) described a thermolabile factor in the plasma of humans and primates that was needed for the *in vitro* phagocytosis by polymorphonuclear cells of serotype B_I but not for serotype B_{III}. It is necessary to evaluate the virulence of isolates recovered from vaginal cultures taken from asymptotically infected women to determine whether mice will exhibit selective susceptibility related to strain virulence.

The recovery of group B streptococci from urine specimens could represent contamination of the clean voided specimens with organisms present on the genital mucosa. Franciosi et al. (7) isolated these bacteria from 50% of urine specimens from husbands of pregnant women with asymptomatic vaginal infections. In a clinical study in progress here, 45% of pregnant women with asymptomatic group B streptococcal vaginal infections also had group B streptococci in the urine. These urines contained fewer than 10^2 organisms per ml (unpublished observations). Although our experimental attempt to produce bacteriuria and pyelonephritis was unsuccessful, vaginal isolates of the group B streptococci (especially serotypes B_I and B_{III})

should be experimentally tested by the water diuresis model before deciding whether these organisms should be considered primary urinary pathogens or opportunists in the chronically ill.

Experimentally the rate of vaginal colonization was similar for pregnant and for virgin mice. Franciosi et al. (7) and Baker et al. (C. J. Baker, D. K. Goroff, S. Alpert, V. A. Crockett, C. Hayes, S. H. Zinner, J. R. Evrard, and W. M. McCormack, *Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother.*, 15th, Washington, D.C., Abstr. 319, 1975) reported that the incidence of asymptomatic vaginal infections among nonpregnant women was 12 and 17%, respectively, which was comparable to carrier rates seen among several other female populations. In mice it was difficult to determine whether infection was experimentally transmitted to newborn mice at delivery because superinfections with gram-negative organisms were frequent (obscuring or eliminating the streptococci). Another observation was the cannibalization of the newborn mice, which may have been promoted through frequent handling of the adult mice or could be an indication of infection of the newborns. In those mice sacrificed immediately after delivery, no group B streptococci were recovered from the lungs or kidneys.

Persistence of the group B streptococci in the oropharynx of suckling mice born of mothers that had been exposed by vaginal colonization also suggested that host defenses in the newborn were not augmented by any maternal immunity transmitted systemically before delivery or through colostrum. In the suckling mice, infection persisted for 14 days. On the other hand, in the older, weaned mice, clearance of the oropharynx occurred in over 50% of the animals by day 14, suggesting that host defense mechanisms may be stimulated. Studies are underway to determine whether any local and/or systemic immunity develops in this latter group of animals.

The production of persistent bacteremia and the colonization of mucosal surfaces without production of disease suggest that mice are good experimental animals for studies on the pathogenesis of invasive group B streptococcal disease. Such a model can be used to characterize the role of the polymorphonuclear leukocytes in controlling the bacteremia and the effectiveness of antibiotic treatment in controlling bacterial proliferation in the bloodstream, as well as the role of humoral plasma or serum factors in host defenses. In addition, the experimental production of mucosal colonizations will permit characterization in mice of the host de-

fenses affected, be it leukocytosis or production of immunoglobulins, and determination of whether antibiotic treatment can effectively eradicate these bacteria.

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